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The Proto-Oncogene c-*maf* Is Responsible for Tissue-Specific Expression of Interleukin-4

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Summary

The molecular basis for the distinctive cytokine expression of CD4+ T helper 1 (Th1) and T helper 2 (Th2) subsets remains elusive. Here, we report that the proto-oncogene c-*maf*, a basic region/leucine zipper transcription factor, controls tissue-specific expression of IL-4. c-Maf is expressed in Th2 but not Th1 clones and is induced during normal precursor cell differentiation along a Th2 but not Th1 lineage. c-Maf binds to a c-Maf response element (MARE) in the proximal IL-4 promoter adjacent to a site footprinted by extracts from Th2 but not Th1 clones. Ectopic expression of c-Maf transactivates the IL-4 promoter in Th1 cells, B cells, and nonlymphoid cells, a function that maps to the MARE and Th2-specific footprint. Furthermore, c-Maf acts in synergy with the nuclear factor of activated T cells (NF-ATp) to initiate endogeneous IL-4 production by B cells. Manipulation of c-Maf may alter Th subset ratios in human disease.

Introduction

CD4+ T helper (Th) cells are not a homogeneous population but can be divided on the basis of cytokine secretion into at least two subsets, termed T helper 1 (Th1) and T helper 2 (Th2) (Mosmann et al., 1986; Paul and Seder, 1994; Seder and Paul, 1994). Th1 cells secrete interleukin-2 (IL-2) and interferon-γ (IFNγ), while Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Both subsets produce cytokines such as tumor necrosis factor and granulocyte/macrophage colony-stimulating factor. There is now abundant evidence that the ratio of Th1 to Th2 cells is highly relevant to the outcome of a wide array of immunologically mediated clinical syndromes including autoimmune, allergic, and infectious diseases. Therefore, the ability to alter ratios of Th1 and Th2 subsets provides exciting therapeutic options. Such manipulation requires an understanding of the mechanisms by which CD4 T helper precursor cells (Thp), which secrete only IL-2, differentiate into Th1 or Th2 effector cells. It is clear that the cytokines themselves are potent Th cell inducers and form an autoregulatory loop (Paul and Seder, 1994; Seder and Paul, 1994). Thus, IL-4 promotes the differentiation of Th2 cells while preventing the differentiation of precursors into Th1 cells, while IL-12 and IFNγ have the opposite effect. Therefore, one possible means to alter Th differentiation is to increase or block the transcription of selected cytokines. Indeed, administration of recombinant IL-4 or antibodies to IL-12 (Racke et al., 1994; Leonard et al., 1995) ameliorate experimental allergic encephalomyelitis, a Th1-driven autoimmune disease, while anti-IL-4 antibodies cure the Th2-mediated parasitic disease Leishmaniasis major (Sadick et al., 1990).

The molecular basis for the tissue-specific expression of IL-4, or any T cell cytokine, has remained elusive. One possibility is the presence of repressor proteins that selectively silence cytokines. Transcriptional silencing has been well documented for bacterial, yeast, and mammalian genes. Examples include Escherichia coli thermoregulation genes (Goransson et al., 1990), yeast α2 mating type genes (Keleher et al., 1988), and mammalian major histocompatibility complex (MHC) class I and T cell receptor (TCR)α genes (Weissman and Singer, 1991; Winoto and Baltimore, 1989). Indeed, early experiments involving injection of IL-2 genomic DNA into Xenopus oocytes suggested the existence of a repressor protein for IL-2 in resting versus activated T cell extracts (Mouzaki et al., 1991). These studies suggested that the absence of IL-2 production in resting T cells was due to proteins that silenced the transcription of IL-2 by interacting with negative elements in the IL-2 promoter.

A second possibility is the existence of Th-selective transactivators. A family of four related transcription factors called nuclear factor of activated T cells (NF-AT) plays a key role in the regulation of cytokine gene expression (Emmel et al., 1989; Flanagan et al., 1991; Jain et al., 1993; McCaffrey et al., 1993; Rao, 1994; Northrop et al., 1994). However, NF-AT family members bind to and transactivate the promoters of multiple cytokine genes, including IL-2 and IL-4 (Rooney et al., 1995b; Szabo et al., 1993; Flanagan et al., 1991; Northrop et al., 1994). Thus, they are not likely to direct Th-specific cytokine transcription. Most, if not all, NF-AT binding sites in cytokine promoter regulatory regions are accompanied by nearby sites that bind auxiliary transcription factors, usually members of the AP-1 family. We and others have shown that NF-AT and AP-1 proteins bind coordinately and cooperatively and are required for full activity of the IL-2 and IL-4 promoters. Different AP-1 proteins (c-Jun, c-Fos, Fra-1, Fra-2, JunB, and JunD) bind to these sites (Rao, 1994; Jain et al., 1993; Boise et al., 1993; Rooney et al., 1995a, 1995b). However, no evidence for the differential recruitment of these AP-1 family members to the IL-2 or IL-4 promoters has been obtained (Rooney et al., 1995a) and, thus, it is unlikely that Th1/Th2 specificity is achieved through selective usage of these proteins.

Recently, a new subfamily of AP-1/CREB/ATF proteins has been described. The v-*maf* oncogene was originally isolated from a spontaneous musculoaponeurotic fibrosarcoma of chicken and identified as the transforming gene of the avian retrovirus AS42 (Nishizawa et al., 1989). v-*maf* encodes a 42 kd basic region/leucine zipper (b-zip) protein with homology to the c-*fos* and c-*jun* oncogenes. Its cellular homolog, the c-*maf* proto-oncogene, has only two structural changes in the coding

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region from v-*maf* and when overexpressed can cause cellular transformation (Kataoka et al., 1993). The Maf oncogene family includes the "big" Mafs, which include c-Maf (isolated from chicken, mouse, and human), MafB (chicken) and a human retina-specific gene Nrl (Swaroop et al., 1992) as well as the "small" Mafs, which include MafK, MafF, MafG and p18, which lack the amino-terminal two-thirds of c-Maf that contains the transactivating domain (Fujiwara et al., 1993; Igarashi et al., 1995b; Andrews et al., 1993; Kataoka et al., 1995). c-Maf and Maf family members form homodimers and heterodimers with each other and with Fos and Jun, consistent with the known ability of the AP-1 proteins to pair with each other (Kerppola and Curran, 1994; Kataoka et al., 1994). The DNA target sequence to which c-Maf homodimers bind, termed the c-Maf response element (MARE), is a 13 bp or 14 bp element that contains a core T-MARE (TRE) or C-MARE (CRE) palindrome, respectively. Little is known about the function of Maf family members, although c-Maf has been shown to stimulate transcription from the Purkinje neuron-specific promoter L7 (Kurschner and Morgan, 1995) and Nrl can drive expression of the QR1 retina-specific gene (Swaroop et al., 1992). The small Mafs have been shown to function as repressors of α- and β-globin transcription when bound as homodimers but are essential as heterodimeric partners with the erythroid-specific factor p45NF-E2 to activate globin gene transcription (Kataoka et al., 1995; Igarashi et al., 1994). MafK overexpression induces erythroleukemia cell differentiation (Igarashi et al., 1995a), while MafB interacts with and represses Ets-1 to inhibit erythroid differentiation (Sieweke et al., 1996). However, there have been no reports implicating c-Maf or Maf family members in the regulation of genes expressed in lymphoid cells or in cytokine gene expression in any tissue.

Here, we present evidence that the c-*maf* proto-oncogene is responsible for the tissue-specific expression of IL-4 in Th2 cells. Furthermore, it is selectively expressed in differentiating and mature Th2 cells and is absent from Th1 cells. To our knowledge, c-*maf* is the only gene identified to date, other than the cytokines themselves, to be differentially expressed in Th subsets.

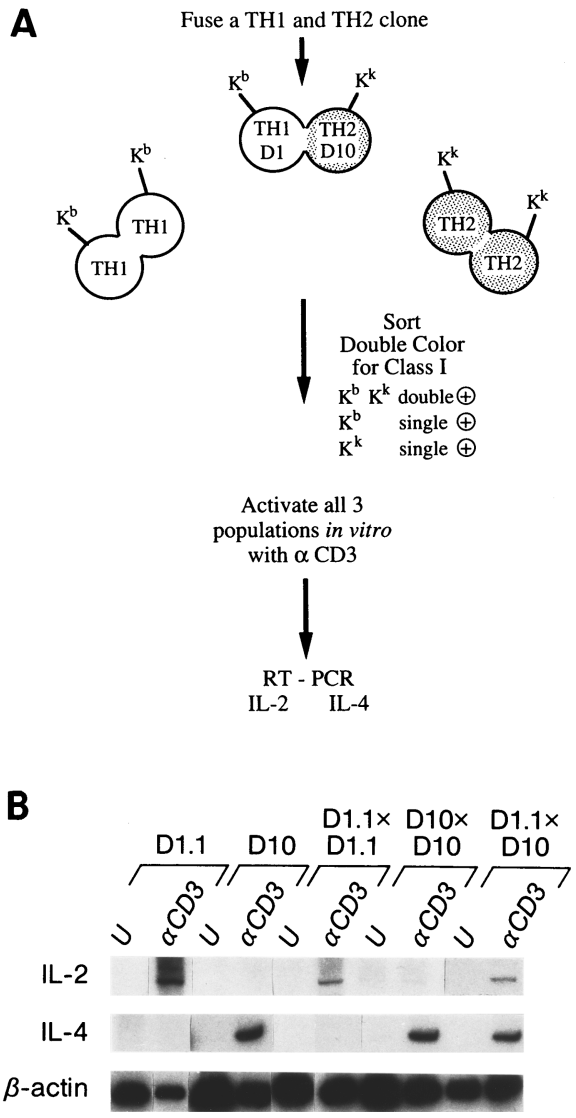

Figure 1. Cytokine Expression Is Not Due to a Repressor

(A) Schema of approach as detailed in Experimental Procedures.

(B) RT–PCR analysis of IL-2 and IL-4 cytokines and control β-actin mRNA produced by the unfused Th1 clone (D1.1), unfused Th2 clone (D10), Th1 and Th2 homokaryons, and Th1–Th2 heterokaryons. Methods are detailed in Experimental Procedures.

Results

Cytokine Specificity Is Due to a Positive Transacting Factor and Not to a Repressor

Tissue specificity can be achieved through the action of repressor or silencer proteins. Thus, it was possible that the IL-2 and IL-4 genes were actively repressed in Th2 and Th1 cells respectively. To test for the existence of repressor proteins, we performed somatic cell fusions between a Th1 (D1.1) and a Th2 (D10) clone and took advantage of polymorphic cell surface MHC class I antigens to sort the fused cells by flow cytometry into three populations, as schematized in Figure 1A. Cells expressing both MHC class I K^b and K^k markers were heterokaryons, while cells expressing only K^b or K^k represented homokaryons and served as controls. The three populations were then stimulated in culture with antibodies to CD3 to activate cytokine gene expression, and RNA was prepared for reverse transcriptase–polymerase chain reaction (RT–PCR) and Northern blot analysis. As shown in Figure 1B, the Th1 and Th2 clones and Th homokaryons transcribed only IL-2 (Th1) or IL-4 (Th2), respectively, while the Th1/Th2 heterokaryons produced both cytokines. The existence of repressor protein(s) should have resulted in the extinction of both cytokines in the heterokaryons. Thus, our conclusion from these experiments was that cytokine specificity was likely mediated by Th-specific positive transacting factors rather than by selective silencer proteins.

Isolation of a Th2-Specific Gene from a cDNA Library Prepared from an Anti-CD3-Activated Th2 Clone

In the course of screening a cDNA library prepared from an anti-CD3-activated Th2 clone, D10, for NF-AT-interacting proteins by the yeast two-hybrid system, we isolated multiple cDNAs, all of which were extremely weak interactors. All cDNAs obtained in this screen were next evaluated for Th-specific expression by Northern blot analysis using a panel of Th1 and Th2 clones. One such cDNA, which was repeatedly isolated, detected transcripts present in Th2 clones (D10, CDC35) but not in either Th1 clones (AR5, OF6, D1) or in a B cell lymphoma, M12 (Figure 2A). Furthermore, the levels of transcripts detected in D10 Th2 cells were substantially increased upon activation by ligation of the TCR with anti-CD3 antibody. No induction of the transcript occurred in Th1 clones upon anti-CD3 treatment. A control probe, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), demonstrated approximately equal loading of RNA in all lanes (Figure 2A). Thus, the expression of this cDNA in the lymphoid lineage appeared to be Th2-specific and sensitive to signals transmitted through the TCR.

To determine whether the expression of this gene was tissue specific and regulated during the course of normal Th cell development, Thp spleen cells were differentiated in vitro (Hsieh et al., 1995). Naive spleen Thp cells were driven along a Th1 or Th2 pathway by treatment with anti-CD3 in the presence of cytokines and anticytokine antibodies (IFNγ and anti-IL-4 for Th1, IL-4 and anti-IFNγ for Th2). Northern blot analysis of differentiating cells harvested at various time-points after stimulation in a primary (day 0–8) and secondary (0–20 hr) response was performed (Figure 2B, bottom) and identification of differentiating Thp cells as Th1 or Th2 was determined by analyzing culture supernatants by enzyme-linked immunosorbent assay (ELISA) for IL-10 (Figure 2B, top) and IFNγ (data not shown). In two experiments, this analysis revealed low level (Figure 2B) or undetectable (data not shown) expression of this cDNA in naive spleen cells at day 0, while in cultures differentiating along a Th2 pathway, substantial induction of transcripts occurred by day 8 in a primary stimulation and by hour 20 in a secondary stimulation. In contrast, no induction occurred in cells being driven along a Th1 pathway. A control probe (GAPDH) showed approximately equal loading of RNA in all lanes (data not shown). The low level of transcripts present in cells being driven along a Th1 pathway likely reflects the presence of residual Th2 cells, since this in vitro differentiation system does not result in a complete shift to one subset. Together, these experiments revealed that the cDNA isolated is selectively expressed in Th2 clones, where it is induced upon T cell activation, and that it is absent from Th1 clones. Furthermore, this gene is induced in normal Thp when they are driven towards the Th2 lineage but is not induced during Th1 development.

The cDNA obtained from the yeast two-hybrid screen was used as a probe to isolate a full-length cDNA from the D10 Th2 cell cDNA library. Sequence analysis revealed that this Th2-specific gene was identical to the c-*maf* proto-oncogene, although the 4.3 kb clone we

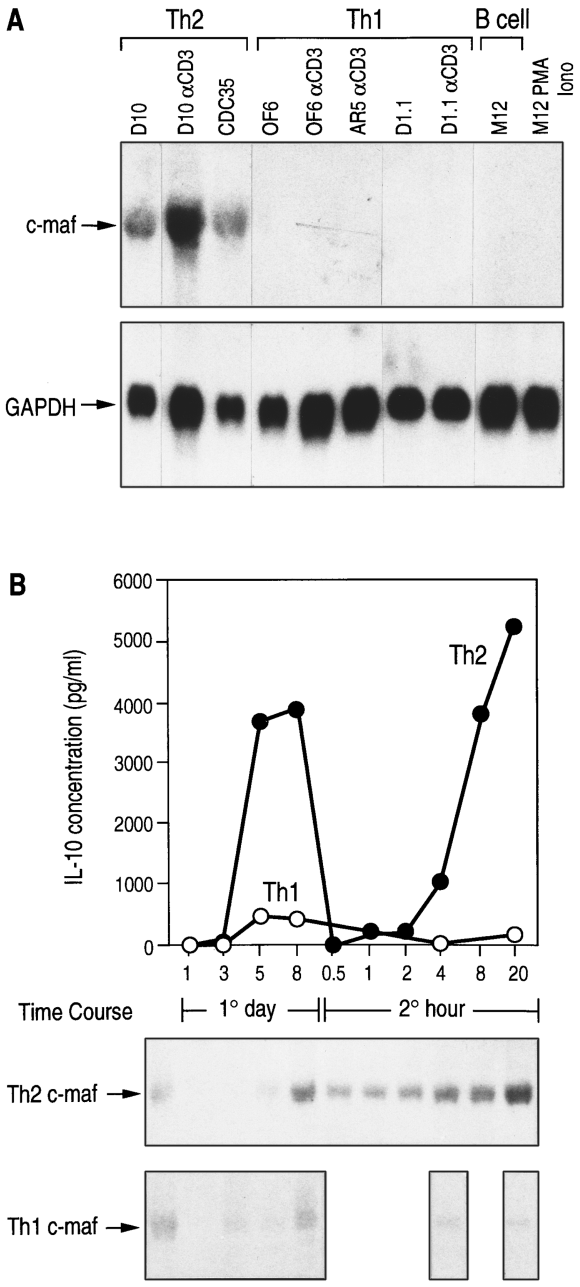

Figure 2. Northern Blot Analysis of the cDNA Clone Isolated from an Activated D10 Th2 cDNA Library

(A) Th2-specific expression in Th clones. Total RNA was isolated from the panel of Th1 and Th2 clones either unstimulated or stimulated with anti-CD3 antibody and from M12 B lymphoma cells and analyzed by Northern blot as described in Experimental Procedures. A control probe specific for GAPDH was used to show equal loading of RNA.

(B) Up-regulation of Th2 cDNA during in vitro differentiation of normal naive spleen cells into Th2 cells. In vitro T cell differentiation was performed as described in Experimental Procedures. Total RNA was isolated from cells harvested at the indicated time-points. Culture supernatant at the appropriate dilution was measured for cytokine (IL-10) production by ELISA to determine differentiation into the Th1 or Th2 lineage. The data are representative of two independent experiments.

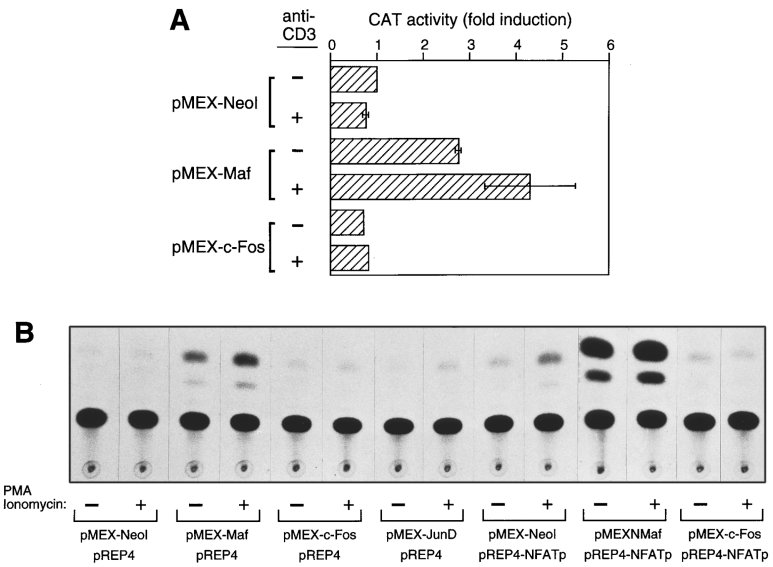

Figure 3. Transactivation of the IL-4 Promoter by c-Maf

AE7 (A) or M12 (B) cells were transiently transfected with a wild-type IL-4 CAT reporter, containing the region (−157 to +68) of the murine IL-4 promoter, and indicated expression vectors. Half of each sample was stimulated 24 hr after transfection with antibodies to CD3. All samples were harvested 48 hr after transfection, and relative CAT activities were determined. (A) is the average of two independent experiments; (B) is one of three independent experiments. See the legend to Table 1 for a description of the expression plasmids used.

obtained had an additional 1.6 kb of 3′ untranslated sequence when compared with previously published isolates (Kerppola and Curran, 1994).

Ectopic Expression of c-Maf in Th1 and B Cells Transactivates the IL-4 Promoter

The identification of this cDNA as a member of the AP-1/CREB/ATF gene family, together with its selective expression in Th2 cells, raised the possibility that c-Maf controlled the tissue-specific transcription of the IL-4 gene. The presence of transcripts encoding c-Maf correlated well with IL-4 expression in Th2 cells and in 3 of 4 transformed mast cell lines examined (data not shown).

Th1 clones and the B lymphoma M12 do not express c-Maf (Figure 2A) nor do they transcribe the IL-4 gene. If c-Maf is a transcription factor critical for controlling IL-4 gene expression, then ectopic expression in these cells should permit IL-4 gene expression. To test this, the full-length c-Maf cDNA was cotransfected with an IL-4 promoter (−157 to +68) reporter plasmid into the Th1 clone AE7. We have previously shown that Th2-specific inducible IL-4 expression can be directed by as little as 157 bp of the proximal IL-4 promoter. In Figure 3A, we show that ectopic expression of c-Maf resulted in substantial activity of the IL-4 promoter in the Th1 clone AE7 after stimulation through the TCR. The fold induction observed was approximately 5-fold over that observed with the control vector alone.

To test more rigorously the ability of c-Maf to transactivate the IL-4 promoter in another non-IL-4-producing cell, we performed the same experiment in the B lymphoma cell line, M12. Normal B cells and B lymphoma cells do not produce IL-4. The results, shown in Figure 3B and Table 1, confirmed our findings in the Th1 clone. Ectopic expression of c-Maf resulted in a dramatic increase in the activity of the IL-4 promoter in unstimulated M12 cells. The fold induction observed, when compared with transfection of a control vector, averaged approximately 50-fold in unstimulated M12 cells. Stimulation of M12 cells with a PMA/Ca^{2+} ionophore, which should result in translocation of NF-ATs to the nucleus and induction of other AP-1 family members (Flanagan et al., 1991; Jain et al., 1993), increased the basal activity of the IL-4 promoter, but a marked induction in promoter activity by c-Maf was still present (average ~25-fold). c-Maf did not transactivate a control reporter driven by NF-AT multimers, demonstrating specificity of transactivation (data not shown). One representative experiment and a summary of three independent experiments are shown in Figure 3B and Table 1.

Table 1. c-Maf Transactivates the IL-4 Promoter Synergistically with NF-ATp

Expression Constructs	PMA Ionomycin	CAT Activity (Fold Induction)[b] Exp. 1	Exp. 2	Exp. 3
Control	−	1	1	1
	+	7.6	1	1.4
c-Maf	−	95	5	18.6
	+	186	7	37
c-Fos	−	2.7	1	0.8
	+	7.6	1.2	1
JunD	−	ND	0.9	0.5
	+	ND	1.4	1.9
NF-ATp	−	14.2	1.6	0.3
	+	41.2	3.5	0.3
c-Maf + NF-ATp	−	136	54	26.3
	+	138	100	54.7
c-Fos + NF-ATp	−	7.4	1.6	3
	+	15.4	1.9	6.1

Summary of three independent transient transfection experiments in M12 cells showing transactivation of the IL-4 promoter by c-Maf and synergy between c-Maf and NFATp. In Experiment 1, 20 μg of cell lysate was incubated for 2 hr. In Experiments 2 and 3, only 5 μg of cell lysate was incubated for 1 hr in order to reveal synergy between c-Maf and NFATp.

[a]Control is pMEX-NeoI.pREP4, c-Maf is pMEX-Maf/pREP4, c-Fos is pMEX-c-Fos/pREP4, JunD is pMEX-JunD/pREP4, NF-ATp is pMEX-NeoI/pREP4-NF-ATp.

[b]Fold induction relative to control, unstimulated.

ND, not done.

As a control for the specificity of c-Maf as opposed to the classic AP-1 family members, the c-Fos and c-Jun proteins were also overexpressed in M12 cells, utilizing the same mammalian expression vector together with the IL-4 reporter plasmid. No IL-4 promoter activity could be achieved through overexpression of these two AP-1 family members. Thus, c-Maf has a unique ability to drive IL-4 gene transcription in M12 B cells. Furthermore, overexpression of c-Maf in the hepatoma cell line HepG2 also resulted in IL-4 promoter transactivation of approximately 100-fold (data not shown). We conclude from these experiments that the provision of c-Maf to c-Maf-negative Th1 or B cells, or to nonlymphoid cells, permits transactivation of the IL-4 promoter.

NF-AT proteins have been shown to be critically important in the regulation of both the IL-4 and IL-2 cytokines. NF-ATp was the first member of this family to be isolated (McCaffrey et al., 1993). Both AE7 and M12 cells have endogeneous NF-ATp protein but do not transcribe IL-4. Although NF-ATp could not, therefore, account for selective IL-4 gene transcription, we wished to test whether overexpression of NF-ATp in unstimulated or stimulated M12 cells would further increase the transactivation of the IL-4 promoter by c-Maf. Overexpression of NF-ATp alone resulted in some modest transactivation of the IL-4 promoter in M12 cells. This transactivation was markedly increased by ectopic expression of c-Maf, an increase that was not additive but synergistic (Figure 3B; Table 1). In contrast, c-Fos overexpression did not further increase the modest transactivation achieved by NF-ATp. We conclude that c-Maf and NF-ATp interact to achieve maximal induction of the IL-4 promoter, the tissue specificity being provided by c-Maf.

Ectopic Expression of c-Maf Activates Transcription of the Endogeneous IL-4 Gene in a B Lymphoma

c-Maf clearly transactivates the IL-4 promoter in transient transfection assays in Th1, B, and nonlymphoid cells. To test whether expression of c-Maf in non-IL-4-producing cells can activate the transcription of endogeneous IL-4, the B lymphoma M12 was stably transfected with c-Maf, NF-ATp, or both. Stably transfected unstimulated M12 cells were plated at the same density and supernatants harvested 24 hr later to measure cytokines by ELISA. Figure 4 shows that M12 cells transfected with c-Maf or NF-ATp alone did not produce measurable IL-4 by ELISA. However, M12 cells stably transfected with both c-Maf and NF-ATp did produce detectable, but low level, IL-4 by ELISA. This was confirmed by RT–PCR of RNA from these cells (data not shown). In contrast, M12 cells transfected with c-Maf and NF-ATp did not produce detectable IL-2 (data not shown). The requirement for both c-Maf and NF-ATp is consistent with the synergistic effect of these factors in the transactivation of the IL-4 promoter noted in the transient transfection experiments in M12 cells. In contrast, stable transfection of M12 cells with junD, an AP-1 family member that can increase IL-4 expression in Th2 cells (Rooney et al., 1995b), alone or together with NF-ATp did not result in IL-4 production. These results demonstrate the essential and selective role of c-Maf in directing tissue-specific endogeneous IL-4 production.

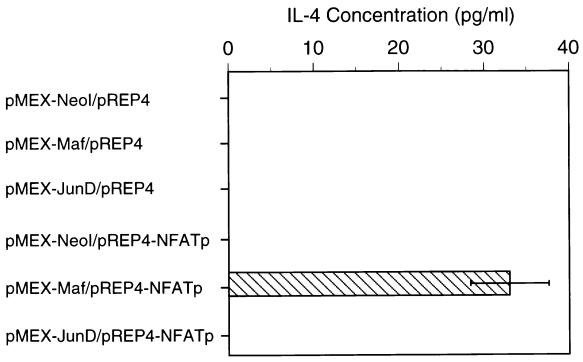

Figure 4. Endogenous IL-4 Production in M12 Cells by Ectopic Expression of c-Maf and NF-ATp

M12 cells were transfected with the indicated expression vectors and cultured in selective media containing G418 (neomycin) and hygromycin. Single or double drug-resistant cells were washed and resuspended at 5×10^5 cells/ml, and 200 μl of supernatant from each sample was subjected to ELISA for cytokine quantitation.

A Site in the IL-4 Promoter Is Footprinted by Extracts from Th2 but Not Th1 Clones

The experiments shown above demonstrated a clear functional role for c-Maf in controlling the tissue-specific expression of IL-4. Furthermore, c-Maf transcripts were expressed in Th2 but not Th1 cells. However, we were unable to detect differences in DNA–protein complexes formed on the IL-4 promoter when using nuclear extracts prepared from Th2 as compared with Th1 cells by electrophoretic mobility shift assays (EMSA) (data not shown). To examine further whether there were proteins, such as c-Maf, in Th2, as opposed to Th1, nuclear extracts that might selectively bind IL-4 promoter sequences, the more sensitive technique of DNAase I footprinting was used. We activated two Th2 clones (D10, CDC35) and two Th1 clones (AE7, S53) by ligation of the TCR with plate-bound anti-CD3 antibody, and nuclear extracts were prepared at time 0 (unstimulated), 2 hr, and 6 hr later. DNAase I footprinting analysis was then performed using an IL-4 promoter fragment (-157 to $+68$). Stimulated extracts from both Th1 and Th2 cells footprinted the two NF-AT sites and the AP-1 site upstream of the distal NF-AT site, as described previously (Rooney et al., 1995b), consistent with the demonstrated function of NF-AT and AP-1 proteins in regulating both the IL-2 and IL-4 promoters (Rooney et al., 1995a, 1995b). Furthermore, inspection of the autoradiograph revealed an area of hypersensitivity on the noncoding strand at residues -28 and -29 when extracts from stimulated Th2 but not stimulated Th1 cells were used (Figure 5A). Unstimulated Th cell extracts did not footprint this region (Figure 5A). The Th2 footprint observed was subtle, but reproducible in two experiments, and is located in a site we had previously demonstrated to be critical for IL-4 promoter activation in Th2 cells (Hodge et al., 1995). A schematic summary of sites occupied in the IL-4 promoter as detected by footprint analysis is shown in Figure 5B. We conclude that a site in the proximal IL-4 promoter, previously shown to be functionally important, is occupied in activated Th2 but not in activated Th1 cells.

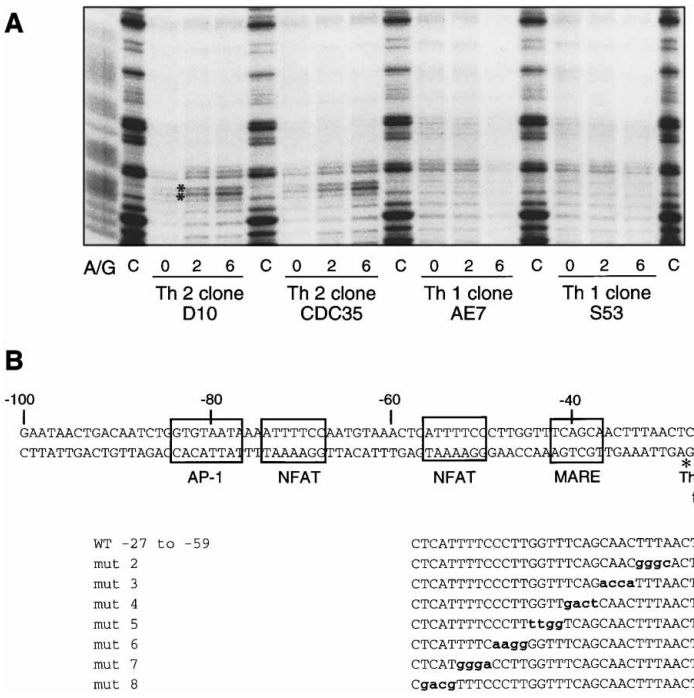

Figure 5. A Th2-Specific Footprint Is Immediately Downstream of the Putative MARE Site in the IL-4 Promoter

(A) DNAase I footprint was performed using nuclear extracts from Th1 (D10, CDC35) or Th2 (AE7, S53) clones harvested at the indicated time-points after stimulation with anti-CD3. Two Th2-specific hypersensitive residues on the noncoding strand of the IL-4 promoter are indicated by an asterisk. We ran 5 lanes of a DNAase I digestion of the IL-4 promoter probe (without nuclear extract) and a Maxam–Gilbert A/G ladder were run next to DNAase I-treated samples.

(B) Schematic representation of the proximal regulatory region of the murine IL-4 promoter and the 4 bp mutants used in Figure 6. The top portion shows the primary sequence of the murine IL-4 promoter. The numbers indicated are relative to the start site of transcription at +1. Asterisks denote the Th2-specific hypersensitive residues seen on DNAase I footprint. The bottom portion shows the sequences of the wild-type (−59 to −28) oligonucleotide and the 4 bp mutants used in EMSA and the functional assays in Figure 6. Altered nucleotides are shown in lowercase bold and correspond to the numbering system shown above.

Recombinant c-Maf Binds to a MARE Site in the IL-4 Promoter

The Th2-specific footprint does not contain a MARE. However, examination of the proximal IL-4 promoter revealed a half c-Maf binding site (MARE) (residues −42 to −37) immediately downstream of the proximal NF-AT site (residues −56 to −51) (Figure 5B) and immediately upstream of the Th2 footprint. We had previously demonstrated that mutation of this site abolished activity of the IL-4 promoter in Th2 cells (Hodge et al., 1995). To determine whether c-Maf bound this site, a truncated c-Maf recombinant protein containing the b-zip domain (amino acids 171–370) was expressed in E. coli, purified on an S-Tag agarose column, and used in EMSA with radiolabeled T-MARE oligonucleotide. The recombinant protein bound well to both a consensus T-MARE oligonucleotide and to a 33 bp oligonucleotide containing the NF-AT site and MARE present in the IL-4 promoter. Furthermore, c-Maf did not bind to an oligonucleotide containing only the NF-AT target sequence to which recombinant NF-ATp bound well. The ability of c-Maf to bind to the IL-4 promoter probe was specific, since in vitro translated c-Jun protein did not bind to this oligonucleotide (Figure 6), nor did recombinant c-Jun protein footprint the MARE site in previously published experiments (Rooney et al., 1995b). The c-Jun protein was functional since it could bind to the consensus MARE oligonucleotide, which contains a core TRE site. We conclude that c-Maf, but not another AP-1 family member (c-Jun), can bind the T-MARE sequence within the proximal IL-4 promoter.

NF-AT proteins interact cooperatively with AP-1 family member proteins to form higher mobility complexes on IL-2 and IL-4 promoter DNA on EMSA (Jain et al., 1993; Rooney et al., 1995b). That they might do so with c-Maf was suggested by the functional studies above. To determine whether c-Maf interacted with NF-AT in the presence of DNA, recombinant NF-ATp and c-Maf proteins were used separately or together in EMSA with the 33 bp oligonucleotide containing both the NF-AT and adjacent MARE sites. Each protein alone bound to IL-4 promoter DNA. Recombinant c-Maf plus recombinant NF-ATp protein produced these complexes and, in addition, formed a higher mobility complex (Figure 6). No higher mobility complex was observed when c-Jun and NF-ATp proteins were used, consistent with the failure of c-Jun to bind this site. We conclude that c-Maf

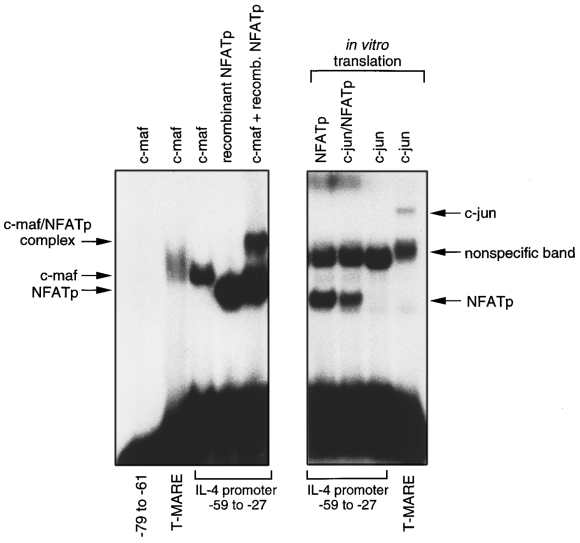

Figure 6. c-Maf, but Not c-Jun, Binds to the Proximal IL-4 Promoter and Forms a Complex with NF-ATp

EMSA was performed using the indicated proteins and labeled ds-oligonucleotides as described in Experimental Procedures. The nonspecific band is seen with the reticulocyte lysate control.

can specifically bind in vitro to a sequence located in the proximal IL-4 promoter, previously shown to be functionally critical in Th2 cells, and that, like other AP-1 proteins, it can interact in vitro with NF-AT proteins.

The Ability of c-Maf to Transactivate the IL-4 Promoter Maps to the MARE and Th2-Specific Footprint

We have previously characterized an essential region of the IL-4 promoter located immediately upstream of the TATA element. High resolution mutagenesis of this 33 bp region (−59 to −27) demonstrated multiple sites required for inducible IL-4 transcription (Hodge et al., 1995). These sites included an NF-AT target sequence, the region footprinted by Th2 extracts, and what we now recognize as a MARE. A series of 4 bp linker-scanning mutants generated across this region were used to map the target sequence utilized by c-Maf in vivo. M12 cells were cotransfected with c-Maf and this series of IL-4 promoter mutants. Mutation of the MARE (mutants 3 and 4), or the site defined by the Th2-specific footprint (mutant 2), abrogated (mutants 2 and 4) or partially abrogated (mutant 3) the ability of transfected c-Maf to drive IL-4 transcription (Figure 7A). A modest effect in reducing c-Maf transactivation was also observed for mutant 8, which disrupts the NF-AT sequence, consistent with the presence in M12 cells of endogeneous NF-ATp and with the synergy between NF-ATp and c-Maf shown above. Mutants 6 and 7 had no significant effect, while mutant 5 had enhanced transactivation ability, consistent with previous observations (Hodge et al., 1995). The transactivation data is consistent with EMSA performed with recombinant c-Maf protein, using as probe an oligonucleotide that contains this 33 bp region and these mutant oligonucleotides as cold competitors (Figure 7B). We conclude from these experiments that c-Maf specifically binds to and transactivates the MARE and that the adjacent Th2-specific element is intimately involved in both the binding and function of c-Maf.

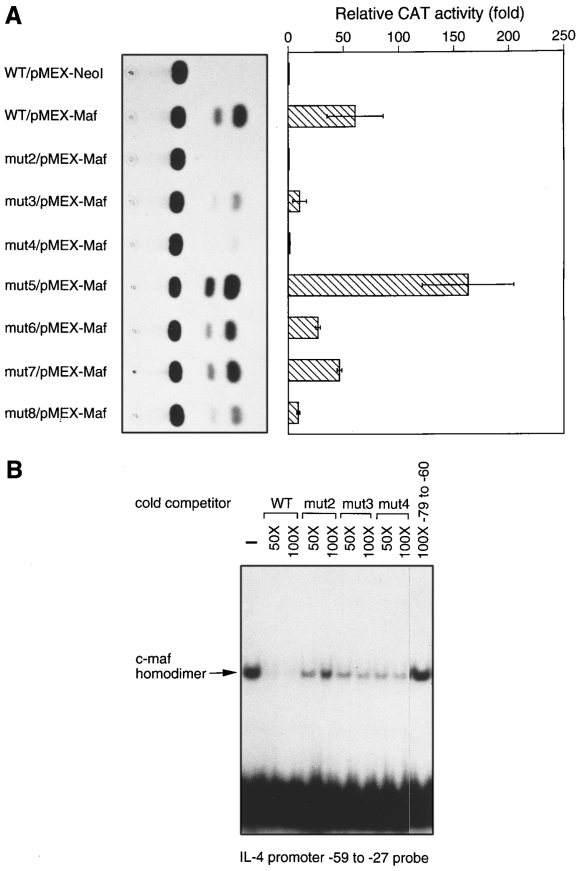

Figure 7. Transactivation of the IL-4 Promoter by c-Maf Maps to the MARE and Th2-Specific Footprint

(A) M12 cells were cotransfected with a c-Maf expression vector and either the wild-type IL-4 CAT reporter or the 4 bp mutants shown in Figure 5B. We incubated 10 μg of each cell lysate at 37°C for 2 hr, and relative CAT activities were determined. The average of three independent experiments and one representative experiment are shown in the top and bottom portions, respectively.

(B) EMSA was performed using recombinant c-Maf and the IL-4 promoter (−59 to −27) probe. Indicated unlabeled ds-oligonucleotides, in 50- and 100-fold molar excess, were used as cold competitors.

Discussion

CD4 Th cells have been subdivided into two subsets on the basis of their pattern of cytokine secretion. However, the molecular basis for this selective expression of cytokines has remained elusive and cannot be attributed to already identified transcription factors such as NF-AT and AP-1 family members known to regulate multiple cytokine genes. Here, we provide strong evidence that the selective expression of the c-*maf* proto-oncogene in Th2 cells accounts for tissue-specific IL-4 expression in these cells. c-Maf is expressed only in Th2 and not in Th1 cells and is induced, together with IL-4, in the former upon transmission of signals through the TCR. Furthermore, c-Maf is induced during the development of normal Thp cells along a Th2 but not a Th1 lineage, and forced expression of c-Maf transactivates an IL-4 promoter in Th1, B, and nonlymphoid cells. Moreover, when both c-Maf and NF-ATp are introduced, B cells can produce endogeneous IL-4. The binding of c-Maf and its transactivation of the IL-4 promoter both map in the proximal IL-4 promoter to a MARE and a site just downstream of the MARE that is occupied by proteins in Th2 but not Th1 extracts. This region of the proximal IL-4 promoter is intimately involved in both the binding and function of c-Maf.

We found no evidence for transcriptional silencing as the mechanism of Th-selective cytokine expression, since somatic cell fusion of a Th1 and a Th2 clone resulted in the production, rather than the extinction, of both the IL-2 and IL-4 cytokines. This is consistent with earlier findings that fusion of a Th1 clone that produced only IL-2 with a cytotoxic T cell clone that produced only IFNγ resulted in the production of both cytokines rather than neither cytokine (Havran and Fitch, 1987). Since the IL-2 silencer described earlier (Mouzaki et al., 1991) could be overcome by proteins present in activated T cell extracts, it is possible that silencers are operative in early Thp but not differentiating T cells. Our results made it more likely that cytokine specificity in

mature Th cells was achieved through the action of tissue-specific activators.

A thorough analysis of the IL-4 promoter in search of such activators has revealed functionally important binding sites for several transcription factors, including NF-AT, CCAAT box binding protein NF-Y, Oct 1, HMG I(Y), AP-1 members, NF-κB, and an as yet unpurified factor termed PCC (Abe et al., 1992; Todd et al., 1993; Szabo et al., 1993; Lederer et al., 1994; Rooney et al., 1995b; Hodge et al., 1995; Chuvpilo et al., 1993). Silencer elements further upstream that bind factors termed NRE have also been described (Le-Weber et al., 1993). None of these factors is expressed in a Th-selective manner. Selective usage of NF-AT proteins by Th1 versus Th2 cells is unlikely, since NF-ATc is present in nuclear extracts of both subsets (M. R. H., unpublished data) and NF-ATp-deficient mice continue to produce both IL-2 and IL-4 (Hodge et al., 1996). Finally, although there has been some evidence that differential regulation of NF-κB may contribute to differences in cytokine transcription between Th subsets (Lederer et al., 1996), the ubiquitous expression of p65 and p50 makes them unlikely candidates for directing Th-specific control. Recently, it has been shown that CCAAT enhancer–binding protein (C/EBPβ) (NF–IL-6), originally isolated by virtue of its ability to regulate IL-6, a cytokine preferentially made by Th2 rather than Th1 cells, can bind to a site that overlaps the MARE. Furthermore, overexpression of C/EBPβ increased IL-4 promoter transactivation in IL-4-producing Jurkat cells (Davydov et al., 1995). It is not known whether C/EBPβ could permit IL-4 gene transcription in Th1 cells. This is unlikely, however, as C/EBPβ is expressed in both Th1 and Th2 cells (I.-C. H., unpublished data). One interesting possibility is that c-Maf and C/EBPβ, both b-zip proteins, may interact to transactivate the IL-4 promoter, the tissue specificity being provided by c-Maf.

The Th2-specific expression of c-Maf described in this report made it an obvious candidate for a transcription factor responsible for dictating Th2-selective IL-4 gene transcription. It should be noted that the site identified in our studies differs from the consensus MAREs previously described. Although it has a consensus core TRE, the IL-4 T-MARE is not palindromic and its flanking sequences are dissimilar from other MAREs. However, the consensus MARE was identified through binding-site selection with recombinant c-Maf protein, an approach that may not reflect the interactions of c-Maf with other factors in vivo (Kataoka et al., 1994). The binding and transactivation data presented in this report provide strong evidence that the IL-4 promoter site is a bonafide MARE. Interestingly, a region of the IL-4 promoter encompassing the MARE and Th2 footprint was shown to be preferentially expressed in Th2 as compared with Th1 cells by Lenardo and colleagues (Bruhn et al., 1993).

One approach to altering Th1/Th2 ratios is to identify genes that are specifically and selectively expressed in Th1 or Th2 cells. Manipulation of such gene products might allow the conversion of these subsets or might enhance the formation of one subset. To date, no Th1 or Th2 genes other than the cytokines have been identified. Two recent reports (Bach et al., 1995; Pernis et al., 1995) have demonstrated that the interferon-β chain is downregulated in Th2 cells during differentiation, but this appears to be a ligand-induced event rather than a consequence of tissue-specific Th cell differentiation. There has also been a report that the Fas ligand receptor is preferentially, although not solely, expressed on Th1 clones rather than Th2 clones (Suda et al., 1995), but FasL expression has not been examined during normal T cell differentiation. c-*maf* is therefore the only Th2-specific noncytokine gene to be identified to date. Analysis of the c-Maf promoter should provide insights into other factors that direct Th2-specific gene expression.

Within the lymphoid lineage, c-Maf is clearly restricted to Th2 cells. The failure of Th1 cells or B cells to transcribe IL-4 is easily explained by the absence of c-Maf. However, outside the lymphoid system, c-Maf is fairly widely expressed in organs such as kidney, testis, placenta, and skeletal muscle. If c-Maf singlehandedly directs IL-4 transcription, why is no IL-4 produced in such nonhematopoietic tissues? One explanation is that there are multiple isoforms of c-Maf, one of which is Th2-selective. Indeed, several members of the maf family have already been identified, some of which (MafK, MafF, p18) may be repressors of c-Maf. Although we have preliminary evidence for an additional c-Maf family member that is ubiquitously expressed (I.-C. H., unpublished data), the cDNA we isolated from Th2 cells does not obviously differ from published isolates aside from additional 3′ untranslated sequence. An attractive possibility is that other forms of maf, such as mafK, may silence the IL-4 promoter in nonlymphoid cells but are displaced in Th2 cells by c-Maf, as has been suggested for p18 and the other small Mafs in regulating β-globin gene expression in the erythroid lineage (Igarashi et al., 1994; Kataoka et al., 1995). A gradient of expression of the various transcriptional activator and repressor maf proteins might exist that ultimately determines whether a given cell will produce IL-4. The ability of c-Maf overexpression to transactivate the IL-4 promoter in HepG2 liver cells is consistent with this explanation. A careful analysis of the relative levels of expression and transcriptional activities of all maf family members in lymphoid and nonlymphoid tissues will be necessary to test this hypothesis. It is also possible that other lymphoid-specific factors, such as NF-ATc, are required for transcriptional activation of Th-specific cytokines. Their absence in nonhematopoietic tissues prevents a Th-selective factor, such as c-Maf, from driving IL-4 gene transcription in these sites. Such factors may be necessary for cytokine transcription but do not direct Th subset–selective transcription. The requirement for both NF-ATp and c-Maf to achieve endogeneous IL-4 production in the B lymphoma provides some evidence in favor of this possibility. Of course, these explanations are not mutually exclusive.

Identification of a Th2-specific transcription factor that controls the production of IL-4 and, hence, the continued formation of Th2 cells may allow the selective manipulation of Th subsets in vivo. Blockade of c-Maf may inhibit Th2 formation and pathogenic immunoglobulin E antibodies in allergic diseases while enhancing the immune response to malignant cells and infectious

agents. In contrast, up-regulation of c-Maf in autoimmunity and in the setting of organ transplantation may permit enhanced Th2 formation at the expense of Th1 cell development with subsequent dampening of the immune response. The predominance of Th2 cells observed in the setting of neonatal tolerance suggests that this may be an attractive approach (Forsthuber et al., 1996). Forced expression of c-Maf in all T cells in transgenic mice, combined with targeted disruption of the c-*maf* gene, should allow us to evaluate such possibilities in vivo.

Experimental Procedures

Cell Culture

Murine Th1 (D1.1, OF6, Ar5, AE7, and S53) and Th2 (D10. G4 and CDC35) clones used in this study have been previously described (Lederer et al., 1994; Barve et al., 1994). A non-IL-4-producing subclone of AE7 (gift of M. Lenardo), as determined by RT–PCR for cytokines, was used. All clones were cultured in RPMI 1640 supplemented with 10% FCS and 10% ConA-stimulated rat splenocyte supernatant, and maintained by biweekly stimulation with appropriate antigen and APCs. M12.4. C3 (M12) is a murine B lymphoma cell line and was cultured in RPMI 1640 supplemented with 10% FCS.

Th Cell Fusion and Cytokine Analysis

The Th1 clone D1.1 (K^d) and the Th2 clone D10 (K^k) were fused according to the suspension cell fusion procedure (Lane et al., 1986). After fusion, the cells were allowed to recover for 8 hr and then double-stained using PE-conjugated anti-K^k and FITC-conjugated anti-K^d antibodies (Pharmingen, La Jolla, CA). Cells were then sorted on the basis of size to distinguish unfused cells from heterokaryons and homokaryons and by fluorescence to identify single-positive and double-positive cells. As indicated in Figure 1A, three populations were sorted for the following: large PE-positive cells (D1.1 × D1.1), large FITC-positive cells (D10 × D10), and large PE-positive and FITC-positive cells (D1.1 × D10). Approximately 5×10^5 cells were obtained for each population. Routinely, 5%–10% of the cells had undergone fusion. Each of these three populations was then split in half, with one half transferred to prerinsed anti-CD3-coated plates and the remaining half to uncoated plates. After 4 hr, the cells were harvested and poly(A)$^+$ RNA–isolated using the MicroFastTrack kit (Stratagene, La Jolla, CA). cDNA was made using the SuperScript kit (GIBCO/BRL, Bethesda, MD), and used for PCR analysis with commercially available primers specific for murine IL-2, IL-4, and β-actin, according to the instructions of the manufacturer (Stratagene). PCR reactions included 0.5 μCi [α-^{32}P]dCTP (3000 Ci/μmol) (NEN Dupont, Boston, MA). PCR products were separated by nondenaturing PAGE and dried and visualized by autoradiography.

Reporters and Expression Vectors

The generation of the wild-type IL-4 CAT reporter, containing an IL-4 promoter fragment from −157 to +68, and its 4 bp mutants was previously described (Hodge et al., 1995). Expression vectors containing the murine full-length cDNAs encoding c-Fos and JunD in the vector of pMEX–NeoI were a gift of R. Bravo. The expression vector for c-Maf was constructed by inserting a full-length cDNA into the SalI site of pMEX–NeoI. The expression vector pREP4–NF-ATp, containing the full-length murine NF-ATp cDNA, was the gift of T. Hoey.

Transfection and CAT Assays

AE7 or M12 cells were transiently transfected by preincubating 0.4 ml of cells, containing 2×10^7/ml AE7 or 3×10^6/ml M12 cells in serum-free RPMI 1640 with 20 μg (AE7) or 5 μg (M12) of each plasmid for 10 min at room temperature. The samples were then electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) set at 975 μF, 280 V, and immediately placed on ice for 10 min. The transfected cells were allowed to recover overnight in complete media and stimulated with plate-bound anti-CD3 antibody (Pharmingen, San Diego, CA), 1 μg/ml in 1× PBS overnight at 4°C or with 50 ng/ml PMA (Sigma, St. Louis, MO) and 1 μM ionomycin (Calbiochem Corporation, La Jolla, CA) for 24 hr. Cell lysate was prepared by freeze–thaw lysis in 0.25 M Tris–HCl (pH 7.8). Equal amounts of protein (between 5–20 μg) were used for CAT assays. CAT assays were performed as previously described (Todd et al., 1993).

For stable transfection, M12 cells were transfected as described above. The transfected cells were allowed to recover in complete media for 48 hr before the addition of neomycin (GIBCO/BRL, Gaithersburg, MD) and hygromycin (Calbiochem Corporation) at a concentration of 400 μg/ml of each antibiotic. The transfected cells were supplemented with fresh media every other day.

In Vitro T Cell Differentiation

Splenic cell suspensions were prepared from 6- to 8-week-old BALB/c mice, cultured in RPMI 1640 supplemented with 10% FCS at a density of 10^6 cells/ml, and stimulated with plate-bound anti-CD3 antibody in the presence of 5 μg/ml anti-IL-4 antibody (11B11) for the Th1 lineage, or 5 μg/ml anti-IFNγ antibody (XMG-1) for the Th2 lineage (Hsieh et al., 1995). We added 50 U/ml IL-2 to all cultures, and 500 U/ml IL4 (Genzyme) to Th2 cultures 24 hr after stimulation. All cells were harvested, washed, and restimulated 7 days after the primary stimulation with plate-bound anti-CD3 antibody.

ELISA for Cytokine Quantitation

All anti-cytokine antibodies were purchased from Pharmingen. ELISA was performed according to the instructions of Pharmingen and as described (Hodge et al., 1996), with the exception that avidin–alkaline phosphatase (Sigma) at 1:500 dilution in PBS/BSA was used in place of avidin–peroxidase. P-nitrophenyl phosphate (GIBCO/BRL) at 4 mg/ml in substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.02% sodium azide [pH 9.8]) was used as substrate.

RNA Isolation and Northern Analysis

Total RNA was prepared by using Trizol (GIBCO/BRL) according to the instructions of the manufacturer. Total RNA (10 μg) from each sample was fractionated on a formaldehyde agarose gel and transferred to a nylon membrane. A 300 bp DraI fragment derived from the 3′ untranslated region of c-*maf* was labeled with [α-^{32}P]dCTP using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed using QuikHyb (Stratagene) according to the instructions of the manufacturer.

Recombinant Proteins and In Vitro Transcription/Translation

The recombinant NF-ATp, containing the Rel domain of human NF-ATp, was a gift of T. Hoey. The expression vector for recombinant c-Maf was constructed by inserting a cDNA fragment encoding amino acid residues 171–370 of c-Maf into the NotI site of pET29 (Novagene, Incorporated, Madison, WI). The truncated c-Maf protein was expressed using T7 polymerase in the BL21(DE3) strain. Cells were induced by the addition of 1 mM IPTG and incubated at 37°C for 3 hr. The induced cells were lysed in 1× Bind/Wash buffer (20 mM Tris–HCL [pH 7.5], 150 mM NaCl, 0.1% Triton X-100) followed by sonication. The c-Maf protein was then purified from the soluble fraction by using the S-Tag Purification Kit (Novagen) according to the instructions of the manufacturer. The in vitro transcription/translation vector TP7-NF-ATp, a gift of T. Hoey, contains a cDNA fragment encoding the Rel domain of human NF-ATp; pGEM-c-Jun was constructed by inserting a full-length murine c-*jun* cDNA into the PstI site of pGEM4. Each plasmid DNA (1 μg) was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate by using the TnT Coupled Transcription/Translation Kit (Promega, Madison, WI).

EMSA

We end-labeled 100 ng of double-stranded oligonucleotides with ^{32}P-dATP (DuPont NEN Research Product, Wilmington, DE) using T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Incorporated, Piscataway, NJ). The labeled ds-oligonucleotides were fractionated on 15%–20% polyacrylamide gels, eluted overnight at 37°C in 1× TE and precipitated in ethanol. Binding assays were performed

at room temperature for 20 min using 0.5 μg of recombinant proteins or 4 μl of in vitro translated products, 500 ng poly(dI–dC), and 20,000 cpm of probe in a 15 μl volume of 20 mM HEPES (pH 7.9), 100 mM KCl, 5% glycerol, 1mM EDTA, 5mM DTT, 0.1% NP-40, and 0.5 mg/ml BSA. The samples were then fractionated in 4% nondenaturing polyacrylamide gel containing 0.5× TBE at room temperature.

Oligonucleotides derived from the murine IL-4 promoter are −59 to −27: 5′-*CTCATTTTCCCTTGGTTTCAGCAACTTTAACTC*-3′; −79 to −60: 5′-*ATAAAATTTTCCAATGTAAA*-3′; and −88 to −61: 5′-*TGG TGTAATAAAATTTTCCAATGTAAA*-3′. The sequence of the MARE oligonucleotide is 5′-*GGAATTGCTGACTCAGCATTACT*-3′. All oligonucleotides were annealed with their respective reverse–complementary strands to form double-stranded oligonucleotides.

DNAase I Footprinting

The preparation of nuclear extracts has been previously described (Rooney et al., 1995b). DNA templates were made by digestion of the IL-4 promoter fragment −157 to +68 with XbaI, kinasing with [α-^{32}P]dCTP and Klenow, digestion with SalI, and gel-purification by PAGE followed by overnight elution and ethanol precipitation. Approximately 100,000 cpm of probe (2–5 ng) was incubated with equal amounts of nuclear extract prepared from different T cell clones in a total volume of 50 ml of 25 mM HEPES (pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 0.05 mM EDTA, 1.0 mM DTT, 10% glycerol, 2% polyvinyl alcohol, and 0.5 mg of poly(dI-dC). We then added 50 ml of 10 mM $MgCl_2$, 5 mM $CaCl_2$ in the presence of varying dilutions of DNAase I. After 2 min of DNAase I digestion, the reactions were stopped by the addition of 100 ml of Stop mix consisting of 200 mM NaCl, 20 mM EDTA, 1% SDS, and 50 mg/ml tRNA. Samples were then phenol-extracted, ethanol precipitated, and resuspended in loading buffer (0.1% bromophenol blue, 0.1% Xylene cyanol, 90% formamide). An equivalent number of counts from each sample was denatured at 90°C for 3 min and loaded onto a 6% polyacrylamide–42% urea gel, run at 80 W for 3 hr, dried, and exposed overnight. Maxam–Gilbert A/G ladders were run alongside the DNAase I-treated samples.

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